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A VALIDATED STABILITY-INDICATING HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF AMOXICILLIN

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يستهدف هذا العمل تشييد طريقة بكروماتوجرافيا السوائل ، متخصصة وبسيطة وسريعة ودقيقة لتحليل الأموكسيسللين ونواتج تحلله المحتملة فى مسحوق العقار. ولقد تم تحليل الأموكسيسللين ونواتج تحلله بإستخدام عمود التراسفير ك١٨ وبإستخدام نظام متحرك يحتوى على ٤٪ كحول ميثيلى فى محلول ٠,٠١ مولار بوتاسيوم فوسفات وحيد القاعدية وترايميثيل أمين (٢ مللى مول) مع ضبط الرقم الهيدروجينى إلى ٣,٦ بإستخدام حمض الفوسفوريك (٠,١ مولار). ولقد رصدت العينات عند طول موجى قدره ٢٤٢ نانوميتر. ولقد تم تكسير الأموكسيسللين فى صورة المسحوق والمحلول بجميع احتمالات ظروف التحلل. وكانت حالات التكسير الإجبارى المستخدمة تشمل دراسة تأثير ضوء النهار والحرارة والرطوبة والتحلل فى وسط حامضى وآخر قاعدى وتأثير الموجات الصوتية والأكسدة. ولقد وجد أن المركب السائد ظهوره من جراء جميع عمليات التكسير القهرى هو حمض بنيسللوك-الأموكسيسللين بينما وجد البنيسيلامين كناتج من عمليات التكسير الصارم. ووجد أن لإستخدام الترايميثيل أمين (كأيون زواجى) تأثير مساعد على إنقاص الحد الكمى لكل من حمض-٦-أمينوبنيسلانك والبنيسيلامين وحمض الكلافيولينك. ولقد نجحت الطريقة فى التطبيق على محقون الأوجمنتين لتقدير كل من الأموكسيسللين وحمض الكلافيولينك.

The purpose of this work is to develop a selective, simple, rapid and accurate liquid chromatographic method for the analysis of amoxicillin and its possible degradation products in bulk drug. Amoxicillin and its degradation products were analyzed on Ultrasphere C18 column, using a mobile system containing 4% methanol in 0.01 M potassium dihydrogen phosphate solution, 2 mM triethylamine, and adjusted to pH 3.6 with 0.1 M phosphoric acid. The samples were monitored at wavelength 242 nm. Amoxicillin was forcedly degraded in powder and solution forms under all possible stress conditions. The forced degradation conditions were including the effect of daylight, heat, moisture, acid-base hydrolysis, sonnication, and oxidation. Amoxicillin penicilloic acid (AMP) was the major degradation product formed under all stress conditions. However, penicillamine (PM) and 6-aminopenicillanic acid (6-APA) were generated under severe stress conditions. The detection and quantification limit of 6-APA, PM and clavulanic acid were enhanced by the use of triethylamine as ion-pair. This method was also applicable for the determination of both, amoxicillin and clavulanic acid in Augmentin parentral.

INTRODUCTION

Amoxicillin; 6-[D-α-amino-α-(p-hydroxyphenyl)acetamido]penicillanic acid, is one of the ß-lactam semisynthetic penicillins.[1] Amoxicillin have been estimated in human plasma by HPLC with on-line post-column derivatization method.[2,3] Most reports described the degradation kinetics of the intact drug, in solution or in combination with other materials, regardless to the applicability of the analytical method as stability indicating assay method.[4-6] However, Valvo *et al.*,[7] found two unusual degradation products in amoxicillin parentral after immediate dissolution of the vial contents in water. These degradation products were (5S, 6R) amoxicillin piperazinedione diasteroisomer, and amoxicilloic acid methyl ester, which were

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detected by HPLC-MS ion spray method. Also, N-formylpenicillamine and penicillamine have been identified as degradation products of some penicillins dissolved in unbuffered solutions of pH 5.[8] Moreover, amoxicillin penicilloic acid, 6-aminopenicillanic acid, and p-hydroxyphenylglycine (p-HPG), were identified in amoxicillin capsules as potential impurities, resulted from non-proper industrial process.[9] These degradation products and impurities were estimated by gradient HPLC method. More specifically, and according to the patent specifications assigned by Bristol-Myers company, only three potential impurities are expected in the active raw material of amoxicillin trihydrate.[10, 11] These impurities include dimethylamine (basic reagent impurity), D-(-)-p-hydroxyphenylglycine (p-HPG), and 6-aminopenicillanic acid (6-APA). The existing steps and limits for each were described. Besides, all possible degradation products of amoxicillin trihydrate or its sodium salt have been reviewed.[12] The United States Pharmacopoeia 23 described an assay method for amoxicillin in pure form and in combination with clavulanic acid.[13] This method was not defined as stability indicating procedure. Described here is a fully validated, developed liquid chromatographic analytical method for the detection and assay of amoxicillin in presence of some possible degradation products. An isocractic HPLC method with Multi-wavelength detection, was designed to fulfill the FDA/ICH requirements,[14-16] regarding the selectivity, linearity, suitability, robustness, ruggedness and sample solution stability. Also, this method was applied for the determination of amoxicillin and clavulanic acid in Augmentin parentral ® (Beecham).

EXPERIMENTAL

Chromatography

The HPLC system consisted of SP8810 pump (Spectra-Physics, San Jose, CA, USA), UV-8 model II Spectrophotometer detector (TSK TOYO SODA, Tokyo, Japan), C-R 3A chromatopac integrator (Shimadzu Co., Ltd., Kyoto, Japan) and a Du Pont column oven (Du Pont Instruments, Wilmington, Delaware, USA) equipped with Rheodyne injector model 7125 (Cotati, CA, USA), and 50 μl sample loop. The chromatographic analysis was performed on a 5 μm, 250 mm x 4.6 mm I.D. Ultrasphere ODS column, from Beckman (Beckman, CA, USA). The mobile phase was prepared by mixing 4 ml methanol, with 96.5 ml 0.01 M potassium dihydrogen phosphate, 0.5 ml 4% triethylamine (99%), and adjusted to pH 3.6 with 0.1 M phosphoric acid. The mobile system was filtered through 0.22 mm Nylon membrane, degassed and pumped at a flow rate of 0.90 ml/min. The data were collected and integrated at 242 nm, while the degraded samples were re-injected using a photodiodarray detector (Tsp Thermo Separation Products, Spectromonitor 5000, connecticut, USA) for peak purity testing.

Chemicals and materials

Methanol and KH_2PO_4, both of HPLC grade from Fisher (Fisher Scientific, NJ, USA). Double-Distilled water in glass was used. Triethylamine (99%) was purchased from Aldrich (Aldrich Chemical Company, Ltd, Gillingham, England). Amoxicillin trihydrate USP reference standard (Rockville, MD, USA). 6-Aminopenicillanic acid and penicillamine were purchased from Sigma (Sigma Chemical Company, MO, USA). Amoxicillin penicilloic acid was prepared by a standard method.[17] Potassium clavulanate as working standard (83%) was from Deva Pharmaceutical Active materials Industry, India. Augmentin intravenous ®, from Beecham Agency in Cairo, Egypt. Each vial labeled to contain 1 g amoxicillin as amoxicillin trihydrate, and 0.2 g clavulanic acid as potassium clavulanate.

Standard solutions

Five Standard solutions having five different concentration levels of amoxicillin trihydrate (AMX), penicillamine (PM), 6-aminopenicillanic acid, and amoxicillin penicilloic acid (AMP) were prepared in 10% aqueous methanol (as under claimed concentration in Table 1. Also different

concentration levels of standard solutions of AMX and clavulanate potassium were prepared (Table 1). For HPLC analysis, 50 μL sample volume was injected three times. The peak area of each peak was plotted versus concentration (μg/ml), and the calibration curves were constructed using least-square regression equation for the calculation of the slope, intercept, and the correlation coefficient.

Forced degradation

Amoxicillin trihydrate was degraded under different stress conditions as follows:

(i) Effect of heat and moisture

Three screw capped brown 1 ml reaction vials were used for this experiment (Kimex, or Alltech, GmbH, USA). The first vial contained 10 mg of amoxicillin trihydrate, and the second vial contained 10 mg AMX with 10 μl water as a source of moisture. Both were kept at 60° for 3 days in a thermostatically controlled hot air oven. The content of the vial was dissolved in water, diluted to 10 ml with the same solvent, and 50 μL was injected for HPLC analysis. Into a third vial an aliquot of 1 ml of standard amoxicillin trihydrate aqueous solution (1 mg/ml) was transferred. This vial was half-inserted into a Block heater of 100° for 5 hours, cooled and 50 μl was analyzed by HPLC for estimation of the remaining amount of amoxicillin trihydrate in addition to the detection and quantification of the degradation products. The samples were diluted if necessary.

(ii) Effect of acid hydrolysis

Into a 1 ml screw capped reaction vial an aliquot of 1 ml of standard amoxicillin trihydrate solution (0.5 mg/ml) was prepared in 0.1 N hydrochloric acid. This vial was half-inserted into a Block heater of 100° for 5 minutes, cooled and a volume of 50 μl was analyzed by HPLC.

(iii) Effect of alkali-hydrolysis

The same procedure as described under acid hydrolysis was followed, but the amoxicillin trihydrate solution was prepared in 0.1 N sodium carbonate. The sample was analyzed after heating times of 15, 30, and 60 minutes at 100°.

(iv) Effect of oxidation

Sample solution of amoxicillin trihydrate (1 mg/ml) was prepared in 1% hydrogen peroxide, as an oxygen rich media, and kept at room temperature for 30 minutes, followed by HPLC analysis of 50 μl. A blank experiment was prepared parallel with the sample treated using only 1% hydrogen peroxide solution.

(v) Effect of sonnication

Into 10 ml volumetric flask, a standard amoxicillin trihydrate aqueous solution (1 mg/ml) was prepared and left to stand in the Ultrasonic bath (50 MHz) for 15, 30, 60 minutes and 2 hours. At each standing time interval, 50 μl was analyzed by HPLC.

(vi) Effect of daylight

Into a 10 cm flat covered colorless glass dish, 30 mg of amoxicillin trihydrate was spread (of about 0.1 mm film thickness). Into another glass dish, 30 mg control sample (amoxicillin trihydrate) was used and wrapped in Aluminium foil. The dishes were kept close to the lab window, away from sunlight, that receives daylight for 60 days. Dissolve 25 mg from each sample powder in 20% aqueous methanol and complete to volume into 25 ml volumetric flask and analyzed by HPLC. Another experiment conducted by subjecting a 30 mg AMX under UV-irradiation, at 254 (5 hours) and 365 nm (5 hours), was investigated.

Generally, the remaining amount of AMX and any generated AMP, 6-APA or PM were calculated from the corresponding calibration line. However, the amount of the generated unknown degradation peaks were calculated from the calibration line of AMX, considering that they have the same response factor according to the ICH Guidelines.[14]

Analysis of Augmetin vials

The Content of one Augmentin vial was (about 1.45 g±0.01), dissolved in 7 ml water, transferred quantitatively to a 100 ml volumetric flask, diluted to volume with water, and mixed well. From this solution, 0.5 ml was diluted to

volume in 100 ml volumetric flask with water, mixed well, and 50 μl was injected for HPLC analysis. Ten vials were analyzed with the same procedure. The vials content were calculated from the corresponding least square equation, considering the dilution factor, clavulanate potassium purity and molar ratios as follow;

Content of amoxicillin = mg/ml calculated from the calibration line x 0.001 x (100/ 0.5) x (365.4 / 419.4),

Content of clavulanic acid = mg/ml calculated from the calibration line x 0.001 x 0.83 x (100/ 0.5) x (199.20/237.24)

RESULTS AND DISCUSSION

Method development

The protocol of the developed method was designed to separate and quantitate the degradation products of amoxicillin as well as to quantitate its potential impurity. Six separate degraded amoxicillin sample solutions were prepared as a result of the effect of heat, sonnication, oxidation, acid-base hydrolysis, and photo-degradation. These samples were injected for HPLC analysis applying several mobile systems, and/or columns. Also, samples containing degraded amoxicillin, were also injected applying several interchangeable chromatographic conditions, until the best resolution. Finally, to obtain the best overall chromatographic conditions, the mobile phase was optimized by examining the effect of pH, column temperature, column type, mobile - flow rate, the percent of methanol to the buffer solution, and the concentration of triethylamine. The optimal chromatographic conditions were achieved as described under the experimental part. Triethylamine concentration was the predominant factor responsible for the bathochromic shifts of the maxima of UV-absorption AMX, 6-APA, and CLV over the other factors.

Linearity and range

A linear response of peak area versus concentration for AMX-$3H_2O$, CLV-K^+, AMP, PM, and 6-APA was observed (Table 1, Figures 1-3). A relatively higher concentration range of AMX was used because it was expected to use a higher concentration to be able for detection and quantification of a small amount of any impurities or degradation products, if any. By this method, even if we inject about 2 mg/ml of AMX, we will be able to quantitate the closest peaks to the major drug, which showed no overlap.

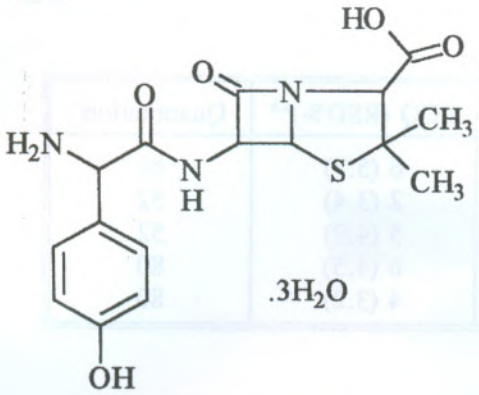
The limit of detection (LOD) was estimated after six repetitive injections. LOD calculated was based on signal-to-noise (S/N) ratio of 3, also from the chromatogram (Table 2). The lower limit of quantification (LOQ) was estimated by satisfying two criteria: first the S/N ratio was not less than 10, and second, the %RSD of peak area of five replicate injections of the LOQ solutions were not more than 6% (Table 1).

Selectivity, precision, and performance parameters

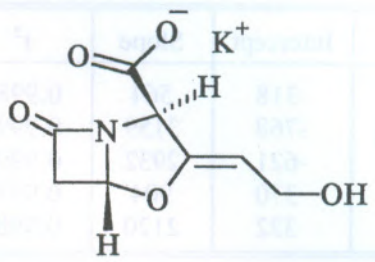
Two separate standard solution mixtures were prepared and each injected six repetitive times. The first standard solution contained AMX-$3H_2O$, PM, AMP and 6-APA (40, 24, 26 and 24 μg/ml respectively), while the second contained AMX-$3H_2O$ and CLV-K^+ (40 and 26 μg/ml respectively). All peaks have shown a complete separation from each other, and from the major AMX peak (Figures 1,2). The system suitability results were calculated according to the general chapter of USP 23 <621> from typical chromatograms.[18] The system was suitable and precise as shown in Table 2.

Peak purity

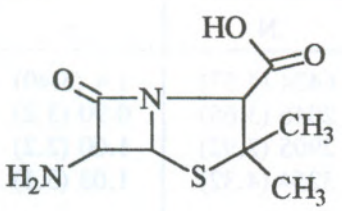
Peak purity is defined as peak similarity index value. All chromatographic peaks of the standard substances were UV-scanned at peak apex, up-slope, downslope, and normalized for estimating the matching percentage. The peak purity index was equal to 1.00, for all compounds.



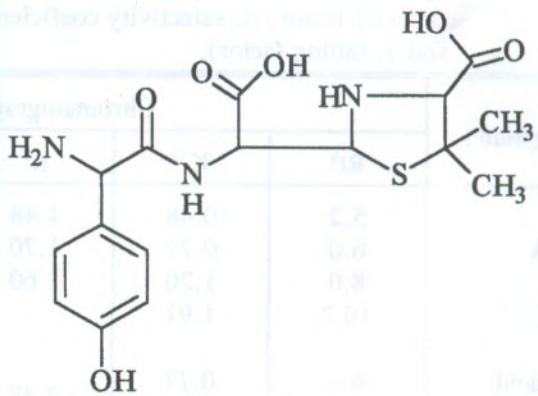
Amoxicillin trihydrate



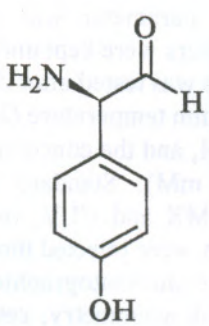
Clavulanate potassium



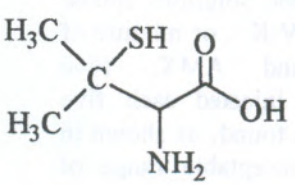
6-Aminopenicillanic acid



Amoxicillin penicilloic acid



D-(-)-p-Hydroxyphenylglycine



Penicillamine

Table 1: Calibration curve data of the investigated compounds.

Compound	Intercept	Slope	r^2	LOD[a]	LOQ (RSD%)[a,b]	Quantitation[a]
CLV-K^+	-318	564	0.9981	2.4	6 (5.7)	80
AMX.$3H_2O_2$	-768	3159	0.9995	1.0	2 (3.4)	52
AMP	-621	2932	0.9993	1.5	5 (4.2)	52
PM	370	904	0.9980	3.0	6 (4.5)	80
6-APA	322	2120	0.9985	1.0	4 (3.2)	80

[a]Concentration, μg/ml
[b]Relative standard deviation of peak area for six determinations.

Table 2: System performance parameters of the investigated compounds (Rt, retention time; K`, capacity factor; α, selectivity coeficient; R, resolution; N, number of theoretical plates; and τ, tailing factor).

Compound	Chromatographic parameters (RSD%)[a]					
	Rt[b]	K`[b]	α[b]	R	N	τ
PM	5.2	0.48	1.48	2.20	6424 (4.57)	1.4 (5.40)
6-APA	6.0	0.71	1.70	2.50	2046 (3.65)	0.90 (3.2)
AMP	8.0	1.20	1.60	3.11	2905 (2.92)	1.00 (2.2)
AMX	10.2	1.91			3254 (4.32)	1.03 (3.1)
CLV acid	6.2	0.77	2.48	6.52	2149 (7.10)	1.19 (7.7)
AMX	10.2	1.91			3254 (4.32)	1.03 (3.1)

[a]Cited data were average of six repetitive injections.
[b]The RSD% values were not more than 2.

Accuracy

The accuracy of the developed method was verified by analyzing placebo solutions spiked with known amounts of CLV-K^+, or mixture of PM, AMP, 6-APA and AMX. Five concentration levels were injected each five times. Since the percentage found, as shown in Table 3, are within the acceptable range of 100% $\pm$5, and the percentage errors were not more than 2.5%, the method is deemed to be accurate.

Robustness

For the evaluation of the method robustness one chromatographic parameter was changed while the other parameters were kept unchanged. The method robustness was tested after changing the pH (3.2–4.2), column temperature (25–50°), percentage of methanol, and the concentration of triethylamine (0.5–4 mM). Standard solution mixture containing AMX and CLV, or AMX, PM, AMP, and 6-APA were injected three times after each change. The chromatographic profile (including k`, Rt., peak asymmetry, resolution, N, and peak width) were calculated and compared with those of the system suitability (Table 2). The results revealed that the method is robust for these small changes

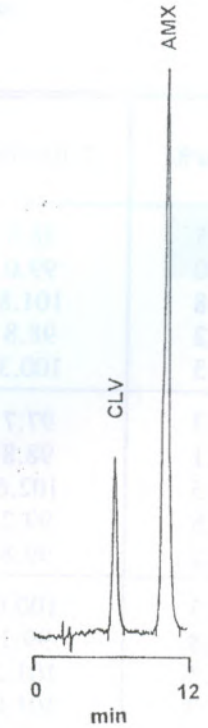


Fig. 1: Representative chromatogram of clavulanate potassium, 26 μg/ml (ClV) and amoxicillin trihydrate, 40 μg/ml (AMX).

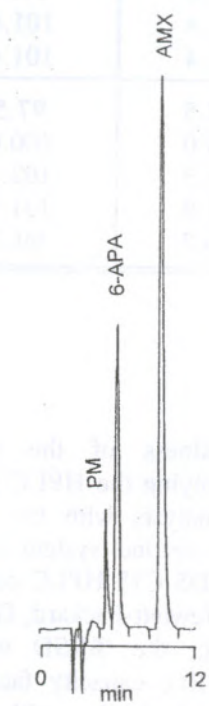


Fig. 2: Representative chromatogram of a standard solution mixture of penicillamine, 20 μg/ml (PM), 6-aminopenicillanic acid, 15 μg/ml (6-APA), and amoxicillin, 20 μg/ml (AMX).

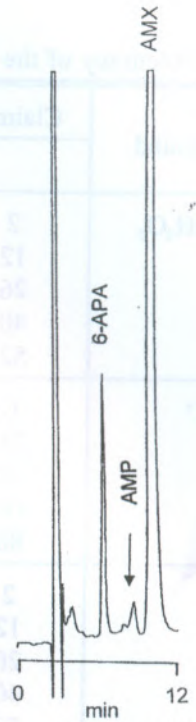


Fig. 3: Chromatogram of amoxicillin (1 mg/ml) solution degraded with hydrogen peroxide 1% for 30 min.

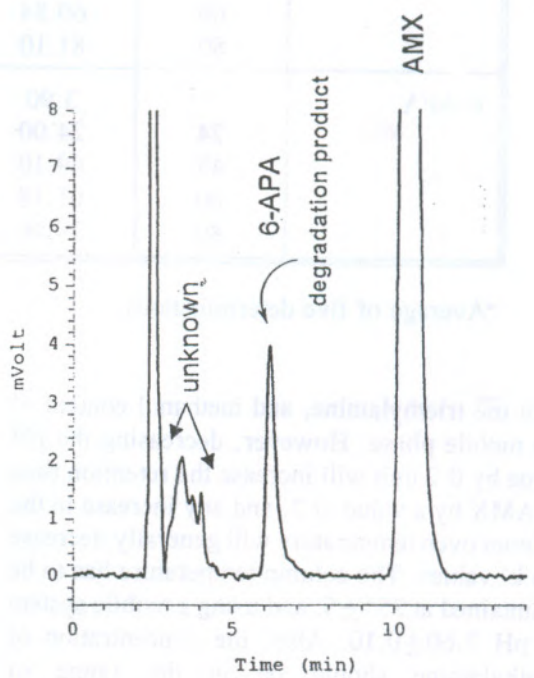


Fig. 4: Chromatogram of photodegraded amoxicillin trihydrate powder dissolved in methanol/water (1:5), 1 mg/mL.

Table 3: Accuracy of the developed method.

Compound	Claimed	Found[a]	Difference	Error%	% Recovery
	(Concentration, μg/ml)				
$AMX.3H_2O_2$	2	1.97	-0.03	1.5	98.5
	12	11.88	-0.12	1.0	99.0
	26	26.47	0.47	1.8	101.8
	40	39.52	-0.48	1.2	98.8
	52	52.16	0.16	0.3	100.3
$CLV\text{-}K^+$	6	5.86	-0.14	2.3	97.7
	26	25.70	-0.30	1.1	98.8
	40	41.02	1.02	2.5	102.6
	60	59.50	-0.50	0.8	99.2
	80	79.83	-0.17	0.2	99.8
AMP	2	2.01	0.01	0.5	100.0
	12	11.90	-0.10	0.8	99.1
	26	26.33	0.33	1.3	101.2
	40	40.42	0.42	1.0	101.0
	52	52.22	0.22	0.14	100.0
PM	6	5.88	-0.12	2.0	98.0
	24	24.10	0.10	0.4	100.4
	48	47.73	-0.27	0.6	99.4
	60	60.84	0.84	1.4	101.4
	80	81.10	1.10	1.4	101.4
6-APA	4	3.90	-0.10	2.5	97.5
	24	24.00	0.00	0.0	100.0
	48	49.10	1.10	2.3	102.3
	60	61.18	1.18	1.9	101.9
	80	79.46	-0.54	0.7	99.3

[a]Average of five determinations.

with the triethylamine, and methanol content of the mobile phase. However, decreasing the pH value by 0.2 unit will increase the retention time of AMX by a value of 2, and any increase in the column oven temperature will generally decrease the k` values. The column temperature has to be maintained at $25° \pm 5$, and using a mobile system of pH 3.60 ± 0.10. Also, the concentration of triethylamine should be in the range of $2mM \pm 0.5$, to avoid any change in the peak area.

Ruggedness

The ruggedness of the method was evaluated by applying the HPLC procedure by two different analysts with the same HPLC system, but the second system was equipped with Hypersil ODS C18 HPLC column (5 μm, 250 x 4.6 mm, Hewlett-Packard, Germany). For amoxicillin peak, the %RSD values of the retention time (Rt), capacity factor (k`), and peak areas obtained with the two

chromatographic systems and operators were not more than 2.0%.

Sample solution stability

Sample solution stability was tested by daily injection of one calibration solution mixture (left at room temperature) containing AMX and CLV, or AMX, PM, and 6-APA. These sample solutions were prepared in glass vial, and injected along three repetitive days. The recovered amount of all substances were about 100% ±2 in the first day. However, the samples analyzed in the third day have shown a content of 100% ±7, without detecting any degradation products because of the low concentrations used. Besides, the intra-day coefficient of variation values were 2.2, 1.5, 1.3, and 2 for the recovered amount of CLV, AMX, PM, and 6-APA, respectively. Also, the inter-day coefficient of variation values were 4.4, 2.1, 4.3, and 7.1 for CLV, AMX, PM, and 6-APA respectively. These high %RSD values may be attributed to the glass adsorption liability of 6-APA and PM.

Forced degradation

The degradation study of AMX was conducted until more than 10% of intact AMX was lost. The results of the effect of heat and hydrolysis in basic media revealed that only 6-APA was detected. The amount of 6-APA detected was not dependent on the presence or absence of moisture. However, amoxicillin penicilloic acid was generated after short heating time (1 min) as a result of ß-lactam cleavage. Boiling in 0.1 N HCl aqueous solution for one minute resulted in more than 50% of 6-APA and less than 50% of AMX, with other unknown degradation products of less than 5%. Also, more than 10% of amoxicillin was lost upon heating at 100° for 20 minutes with 0.1 N NaOH. However, the compounds showed an extensive degradation profile upon just standing, for 5 minutes, in 1% hydrogen peroxide media (Fig. 3). Also, sonnication of the aqueous solution of AMX for more than 30 min, showed less than 6% loss, most of these percentage was in form of 6-APA. Photodegraded AMX solution, showed less than 3% loss as 6-APA and 1% in form of unknown peaks (Fig. 4). When the compound was subjected to daylight for 60 days and the experiment was conducted under UV-light, it showed the same results. In all cases AMP was the major degradation product generated after very short time (1 minute), in addition to some resolved unknown peaks. Besides, penicillamine was only detected if AMX boiled with 1 N HCl for 10 minutes. From these stress experiments, we can conclude that amoxicillin trihydrate is very sensitive towards oxidation and acid hydrolysis, and the dissolution of any AMX powder is not recommended with the aid of Ultrasonic bath cleaner.

This method is not able to detect N,N-dimethylaniline (one of the potential impurities), however, for quality control purpose, it should be simply estimated by any of the reported gas chromatographic methods.[19,20]

Analysis of Augmentin parentral

The developed method was applied for the determination of AMX and CLV in Augmentin parentral. The average percentage recovery of AMX and CLV were, 99.99% ±1.78, and 100.40% ±3.94, respectively (Table 4). The labeled content of the used clavulanate potassium was 83%, and the rest was as water (estimated by Karl Fisher titration method). This was considered in the calculation of the recovered amount of CLV acid.

Conclusion

The developed HPLC method is suitable for separation of some of the degradation products of amoxicillin, in addition to the capability of the method to attribute the hazardous environmental factors or in process of dosage form production, that might affect the drug content. Amoxicillin is preferentially degraded via ß-lactam cleavage to its penicilloic acid, which is then further degraded to 6-APA, PM or any other unknown product.

Table 4: Determination of clavulanic acid and amoxicillin in Augmentin vials[a].

Vial #	Found, mg/vial[b]		% Recovery	
	CLV acid	AMX	CLV acid	AMX
1	211.1	982.4	105.5	98.2
2	199.2	975.5	99.6	97.5
3	203.5	977.3	101.7	97.7
4	200.0	990.6	100.0	99.0
5	200.2	1015.6	100.1	101.5
6	186.2	1024.4	93.1	102.4
7	207.8	1010.7	103.9	101.0
8	203.4	1017.5	101.7	101.7
9	207.9	997.1	103.9	99.7
10	189.6	1008.8	94.8	100.9
Average	200.89	999.99	100.4	99.99
SD	7.899571	17.82311	3.94	1.78
RSD%	3.932287	1.782329	3.93	1.78

[a]Claimed content, clavulanic acid 200 mg, and amoxicillin 1000 mg per vial.
[b]Average of 4 injections.

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